environmental microbiology

Environmental Microbiology (2012) **14**(9), 2361–2378 doi:10.1111/j.1462-2920.2012.02804.x

Structuring of bacterioplankton communities by specific dissolved organic carbon compounds

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Summary

The main role of microorganisms in the cycling of the bulk dissolved organic carbon pool in the ocean is well established. Nevertheless, it remains unclear if particular bacteria preferentially utilize specific carbon compounds and whether such compounds have the potential to shape bacterial community composition. Enrichment experiments in the Mediterranean Sea, Baltic Sea and the North Sea (Skagerrak) showed that different low-molecular-weight organic compounds, with a proven importance for the growth of marine bacteria (e.g. amino acids, glucose, dimethylsulphoniopropionate, acetate or pyruvate), in most cases differentially stimulated bacterial growth. Denaturing gradient gel electrophoresis 'fingerprints' and 16S rRNA gene sequencing revealed that some bacterial phylotypes that became abundant were highly specific to enrichment with specific carbon compounds (e.g. *Acinetobacter* sp. B1-A3 with acetate or *Psychromonas* sp. B3-U1 with glucose). In contrast, other phylotypes increased in relative abundance in response to enrichment with several, or all, of the investigated carbon compounds (e.g. *Neptuniibacter* sp. M2-A4 with acetate, pyruvate and dimethylsulphoniopropionate, and *Thalassobacter* sp. M3-A3 with pyruvate and amino acids). Furthermore, different carbon compounds triggered the development of unique combinations of dominant phylotypes in several of the experiments. These results suggest that bacteria differ substantially in their abilities to utilize specific carbon compounds, with some bacteria being specialists and others having a more generalist strategy. Thus, changes in the supply or composition of the dissolved organic carbon pool can act as selective forces structuring bacterioplankton communities.

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Introduction

The ocean contains one of earth's largest bioactive pools of carbon (i.e. dissolved organic carbon; DOC) and bacteria play a key role in assimilating and transforming this source of reduced carbon (see for example Hansell, 2002). Although a majority of marine DOC consists of refractory compounds that are slowly decomposed by microorganisms, some labile compounds that are found at low nanomolar concentrations, such as glucose or amino acids, are known to account for up to 10–30% or more of the daily organic carbon uptake by bacteria (Kirchman, 2003). To utilize DOC, a great diversity of uptake mechanisms and metabolic pathways for different carbon compounds have evolved among phylogenetically diverse bacteria (Hopkinson and Barbeau, 2011; Sun *et al.*, 2011). Additional characteristics, such as the substrate affinity or efficiency of carbon processing, may also vary substantially among bacterial taxa, critically suggesting that the quality of available compounds could be a strong selective force on bacterioplankton community composition.

The recognized importance of microbial DOC recycling in the ocean has resulted in numerous reports revealing important patterns in the use of different organic matter compounds among marine bacteria, primarily at the level of major bacterial groups but also at the level of specific phylotypes (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Covert and Moran, 2001; Kisand *et al.*, 2002; Pernthaler *et al.*, 2002; Pinhassi and Berman, 2003; Kirchman *et al.*, 2004; Teira *et al.*, 2004; Vila *et al.*, 2004; Elifantz *et al.*, 2005; Mou *et al.*, 2008; McCarren *et al.*, 2010; Alonso-Sáez *et al.*, 2012). For example, among the Alphaproteobacteria, members of the *Roseobacter* clade show high uptake of monomers like glucose and amino acids (Cottrell and Kirchman, 2000; Alonso and Pernthaler, 2006; Alonso-Sáez and Gasol, 2007), while bacteria in the ubiquitous SAR11 clade likely take advantage of highly diluted monomeric compounds (Giovannoni *et al.*, 2005; Malmstrom *et al.*, 2005). Several Gammaproteobacteria, on the other hand, seem to have a preference for elevated concentrations of easily assimilated carbon compounds and inorganic nutrients (Eilers

et al., 2000; Pinhassi and Berman, 2003; Alonso-Sáez and Gasol, 2007; Mou *et al*., 2007). Studies of the variability of bacterial populations in time and space also indicate the role of resources in determining population dynamics, where, for example, the appearance of *Roseobacter* clade bacteria and Flavobacteria has been linked to the organic matter released during phytoplankton blooms (González *et al*., 2000; Pinhassi *et al*., 2004). Taken together, these reports document that an understanding of factors that govern the abundance of particular bacterial species and their resource preferences is basic to understanding how transformations of DOC are regulated in the water column (Kujawinski, 2011).

Studies of the biodiversity of aquatic microbial assemblages have revealed a large richness of bacteria in any given environment, although only a minor portion of the taxa is abundant at each point in time (Pedrós-Alió, 2012). Currently, important efforts are being made to understand the nature of bacterial communities and their potential for responding to natural or human-imposed disturbances on different spatial and temporal scales (Allison and Martiny, 2008). Whether bacterial populations making up particular communities are generalists with respect to their demand for organic and inorganic nutrients or if they are specialists with narrowly defined requirements could have important consequences for determining ecosystem functioning (Mou *et al*., 2008). Moreover, the distribution of life strategies among extant populations possibly affects the resilience of communities, and, for example, a community consisting of specialists could be more sensitive to disturbances than a generalist community (Allison and Martiny, 2008; Shade *et al*., 2011).

Environmental forcing (e.g. changes in nutrient supply) typically generates relatively rapid changes in bacterial community composition (Kirchman *et al*., 2004; Pinhassi *et al*., 2004; Mou *et al*., 2007; Allison and Martiny, 2008). Such compositional changes are frequently accompanied by changes also in ecosystem processes like metabolic activity, e.g. bacterial secondary production, indicating different functional roles for different populations (Kirchman *et al*., 2004; Judd *et al*., 2006; but see also Comte and del Giorgio, 2011). Still, ecosystem processes may also remain stable despite changes in community composition, indicating functional redundancy among bacterial populations (Fernandez *et al*., 2000; Comte and del Giorgio, 2011). Moreover, the development of similar communities in response to different environmental conditions argues that bacterial generalists can also be important players in the cycling of DOC (Langenheder *et al*., 2005; Mou *et al*., 2008; Lekunberri *et al*., 2012). So far, no clear patterns have emerged as to when and where populations responsive to disturbance are either resource specialists or generalists and how this affects ecosystem functioning. It would thus be desirable to better resolve the link between the development of specific bacterial populations and changes in the organic matter field (Allison and Martiny, 2008; Comte and del Giorgio, 2011).

The aim of this study was to investigate how different chemically characterized low-molecular-weight organic compounds, with a proven importance for the growth of marine bacteria, act as a selective force on the composition of marine bacterioplankton assemblages. We carried out a total of nine enrichment experiments, using both newly collected unfiltered seawater (USC) and aged seawater dilution cultures (ASDC). The experiments were carried out in three different seas that differ substantially in trophic status, salinity and bacterioplankton composition. Carbon compound enrichments consisted of acetate, pyruvate, dimethylsulphoniopropionate (DMSP), glycolate, glucose and amino acids. These structurally different compounds were selected since previous studies show that they can each account for significant portions of the daily uptake of organic carbon by bacteria (Fuhrman, 1987; Suttle *et al*., 1991; Rich *et al*., 1996; Obernosterer *et al*., 1999; Skoog *et al*., 1999; Ho *et al*., 2002; Simó *et al*., 2002; 2009; Kirchman, 2003; Zubkov *et al*., 2008). We monitored bacterial growth and the composition of the communities that developed following enrichment with each of the different carbon compounds. Our null hypothesis was that the final bacterial assemblages in different enrichment cultures run in parallel would be qualitatively similar (i.e. with no major differences in presence/absence of taxa or in relative abundances). Our results indicate that changes in the availability of specific carbon compounds can substantially influence the composition of marine bacterioplankton assemblages by selectively favouring the growth of particular bacteria.

Results

Bacterial growth in aged seawater dilution cultures

We carried out the dilution cultures with aged seawater and inocula of freshly sampled natural bacterioplankton assemblages to investigate the response of particular bacteria to enrichment with specific carbon compounds and without interference from flagellate predation (Table 1). In nearly all Mediterranean Sea and Baltic Sea experiments, little growth was observed in the controls without enrichment (Fig. 1), indicating that ageing of seawater had resulted in a substantially reduced pool of labile DOC. Following enrichment with specific carbon compounds, bacterial growth yields of $1–2 \times 10^6$ cells per millilitre were typically observed within 3–5 days. In most experiments, amino acids resulted in the fastest bacterial growth response and the highest yields, with maximum abundances reached already within 2–3 days (Fig. 1).

Table 1. Summary of the aged seawater dilution cultures (ASDC) and unfiltered seawater cultures (USC) performed in the Mediterranean Sea, Baltic Sea and Skagerrak (North Sea).

Experiment	Sampling location	Coordinates	Date	*In situ* Temp (°C)	Incubation Temp (°C)	Type of culture	Carbon compound enrichments[a]
MED1–ASDC	Mediterranean Sea	41°40′N, 02°48′E	22 March 2004	13	13	ASDC	Ace, Pyr, AA, Glu, DMSP
MED2–ASDC	Mediterranean Sea	41°40′N, 02°48′E	25 May 2004	17	17	ASDC	Ace, Pyr, AA, Glu, DMSP
MED3–ASDC	Mediterranean Sea	41°40′N, 02°48′E	19 July 2004	24	24	ASDC	Ace, Pyr, AA, Glu, DMSP
BAL1–ASDC	Baltic Sea	56°37′N, 16°44′E	17 May 2005	12	12	ASDC	Ace, Pyr, AA, Glu
BAL2–USC	Baltic Sea	56°39′N, 16°24′E	17 September 2007	20	20	USC	Ace, Pyr, AA, Glu, Gly
BAL3–USC	Baltic Sea	56°39′N, 16°24′E	8 October 2007	12	12	USC	Ace, Pyr, AA, Glu, Gly
BAL4–ASDC	Baltic Sea	56°39′N, 16°24′E	27 November 2007	10	15	ASDC	Ace, Pyr, AA, Glu, Gly
BAL4–USC	Baltic Sea	56°39′N, 16°24′E	27 November 2007	10	15	USC	Ace, Pyr, AA, Glu, Gly
SKA1–USC	Skagerrak	58°12′N, 11°18′E	6 November 2008	10	15	USC	Ace, Pyr, AA, Glu, Gly

a. Abbreviations denote: acetate (Ace), pyruvate (Pyr), amino acids (AA), glucose (Glu), dimethylsulphoniopropionate (DMSP) and glycolate (Gly).

Bacterial growth in unfiltered seawater cultures

Cultures with newly sampled and unfiltered seawater were used to determine the effect of specific carbon compound additions on the bacterioplankton community when basically the entire microbial food web (including grazers) and a recently produced natural DOC pool were present. Bacterial abundance at the beginning of the USC experiments ranged 2–4 $\times$ 10^6 bacteria per millilitre (Fig. 2). In experiments BAL2–USC and BAL3–USC, bacterial abundance in the enrichments remained similar to the controls (Fig. 2A and B), suggesting that grazing pressure maintained bacteria at relatively stable abundances. Nevertheless, and as compared with the controls, leucine incorporation rates increased more than five- or twofold in the amino acids and acetate or glucose and pyruvate treatments respectively (Fig. 2A and B, insets). In contrast, in experiments BAL4–USC and SKA1–USC (Fig. 2C and D), bacterial abundance was dynamic, with glucose and acetate resulting in the highest peak abundances (in BAL4–USC also together with pyruvate). Bacterial abundance in the glycolate enrichments was similar to the controls. After 2–3 days of growth, and after sampling for bacterial community DNA and production, numbers rapidly decreased in these two experiments, probably due to flagellate grazing.

Denaturing gradient gel electrophoresis (DGGE) analysis of community structure

We carried out nine experiments, comprising a total of 54 duplicate seawater enrichment cultures (i.e. a total of 108 bottle incubations). We did not detect any specific patterns in the number of phylotypes among treatments, but there was a general tendency towards a lower number of bands/phylotypes in the Baltic Sea experiments (for detailed description of DGGE banding patterns and phylotype distributions, see legends to Figs S1 and S2). Dendrogram analysis showed that in 30 of the duplicate

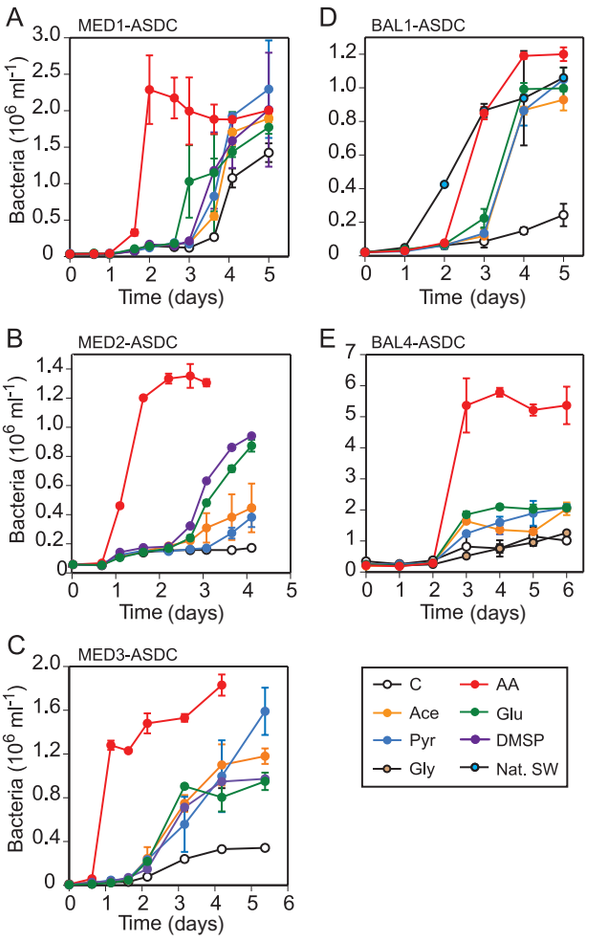

Fig. 1. Growth of bacteria in aged seawater dilution cultures (ASDC) after enrichment with specific carbon compounds. Experiments in the Mediterranean Sea: (A) MED1–ASDC, (B) MED2–ASDC, (C) MED3–ASDC. Experiments in the Baltic Sea: (D) BAL1–ASDC, (E) BAL4–ASDC. Abbreviations in the keys are: controls without enrichment (C), acetate (Ace), pyruvate (Pyr), glycolate (Gly), amino acids (AA), glucose (Glu), dimethylsulphoniopropionate (DMSP), and natural seawater (Nat. SW).

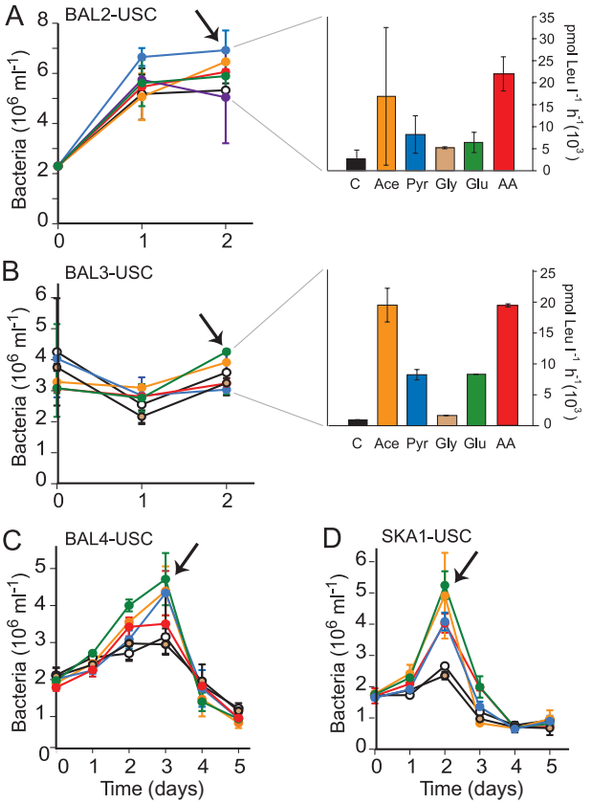

Fig. 2. Bacterial abundance and leucine incorporation rates in unfiltered seawater cultures (USC). Experiments were: (A) BAL2–USC, (B) BAL3–USC, (C) BAL4–USC, and (D) SKA1–USC. Controls without enrichment (C), acetate (Ace), pyruvate (Pyr), glycolate (Gly), glucose (Glu) or amino acids (AA). Error bars denote standard deviations of samples from duplicate bottles. In experiment BAL4–USC, error bars denote standard deviations of triplicate measures from one of the duplicates. Keys and abbreviations same as in Fig. 1. Black arrows indicate the time when DNA samples for phylogenetic analysis were collected; at the same time, the leucine incorporation assays for experiments BAL2–USC and BAL3–USC were performed.

cultures, the duplicates clustered together, indicating that the banding pattern was similar (Figs 3 and 4). In 10 cases, the duplicates formed clusters with other treatments because they shared several phylotypes (e.g. pyruvate, amino acids and glucose in Fig. 3D). Finally, in 14 cases, the duplicates deviated considerably due primarily to the presence of different bands in different duplicates and/or due to substantial differences in the intensity of shared bands between duplicates (e.g. DMSP or acetate in Fig. 3B). Still, even in such cases several shared bands were similar between duplicates (e.g. DMSP or acetate in Fig. S1B).

Cluster analyses of the bacterial community composition in the enrichment experiments using ASDC in Mediterranean Sea showed that the community composition differed between carbon compounds (Fig. 3). Notably, several *Alteromonas* phylotypes responded to only a few carbon compounds (e.g. phylotype M1-A4 was only found with amino acids, Fig. S1A). As seen in the dendrograms (Fig. 3A–C), the bacterial assemblages in replicate cultures of the control, pyruvate and acetate treatments clustered together and showed relatively similar DGGE banding patterns ('fingerprints') in two out of three experiments (Figs 3A–C and S1A–C). Also for most of the other treatments, there were individual bands that were found in both replicates of a specific treatment, although the general cluster analysis did not group the duplicates together. However, for DMSP there were considerable differences between duplicates in all three experiments. It remains unclear why differences occurred repeatedly precisely in this treatment, but similar differences between duplicate cultures have previously been reported by Vila-Costa and colleagues (2006), analysing phylotypes responsive to dimethyl sulphide enrichments in Gulf of Mexico seawater cultures.

Baltic Sea experiments were carried out using both ASDC and USC. Experiment BAL1–ASDC revealed a relatively low number of dominant phylotypes (Fig. S1D), with cluster analysis revealing a good reproducibility between duplicates for acetate, control and natural seawater compared with the other treatments (Fig. 3D). The most striking observation in this experiment was the strong dominance of *Acinetobacter* phylotype B1-A3, which was unique for the acetate enrichment (Fig. S1D, Table 2). The DGGE fingerprints of experiments BAL2–USC and BAL3–USC were remarkably similar (Fig. S2), considering that they were carried out 1 month apart, both with *Psychromonas* phylotype B3-U1 strongly dominating in the glucose enrichments. Hence, cluster analysis showed that, in these experiments, the glucose duplicates grouped together and were well separated from the other enrichments (Fig. 4A and B). In experiment BAL4–USC, many of the phylotypes that were present in the *in situ* sample were also found in the enriched cultures (Fig. S2C), and the glucose and amino acids enrichments were those that triggered the most pronounced changes in the community structure (see cluster analysis in Fig. 4C). Finally, in experiment BAL4–ASDC (Figs 3E and S1E), the majority of phylotypes were present in most of the treatments; the main differences were related to band intensity rather than to band presence/absence.

The Skagerrak experiment was carried out using the USC approach (SKA1–USC). In this experiment each carbon compound triggered the growth of a unique combination of phylotypes, and analysis of the DGGE fingerprints revealed a tight clustering of duplicates for glycolate, pyruvate, amino acids and glucose, but not for acetate (Fig. 4D). While some phylotypes were found in

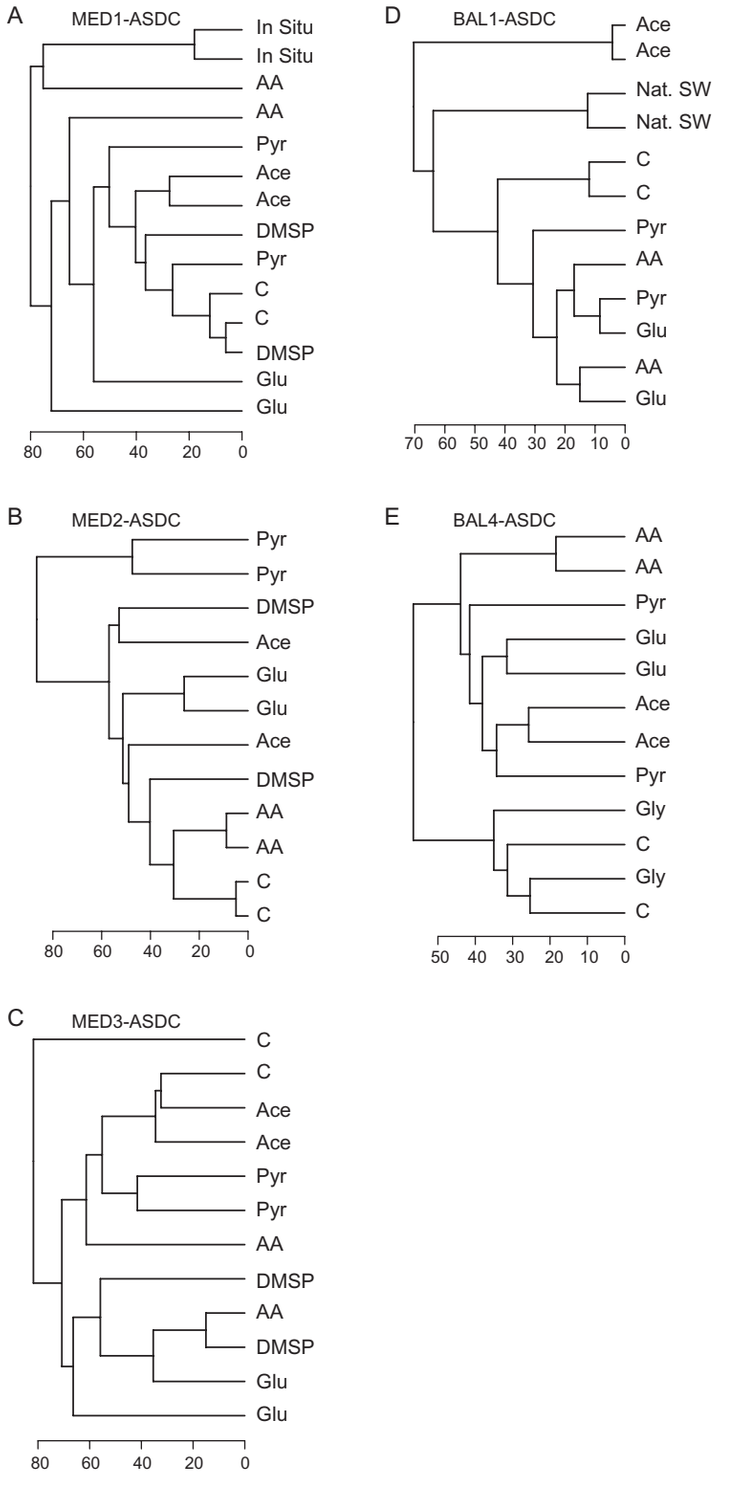

Fig. 3. Cluster analysis of bacterial community composition in the aged seawater dilution culture (ASDC) enrichment experiments. (A) MED1–ASDC, (B) MED2–ASDC, (C) MED3–ASDC, (D) BAL1–ASDC and (E) BAL4–ASDC. The dendrograms are based on the analysis of the DGGE images presented in Fig. S1. Controls without enrichment (C), acetate (Ace), pyruvate (Pyr), dimethylsulphoniopropionate (DMSP), glucose (Glu) or amino acids (AA).

several treatments, the DGGE banding patterns of each treatment was different (Fig. S2D). For example, in the pyruvate treatment three dominant bands were observed, while only two of these bands were present with amino acids; and while the glucose treatment contained numerous bands, the glycolate treatment was dominated by the conspicuous *Rhodobacter* phylotype S1-U2.

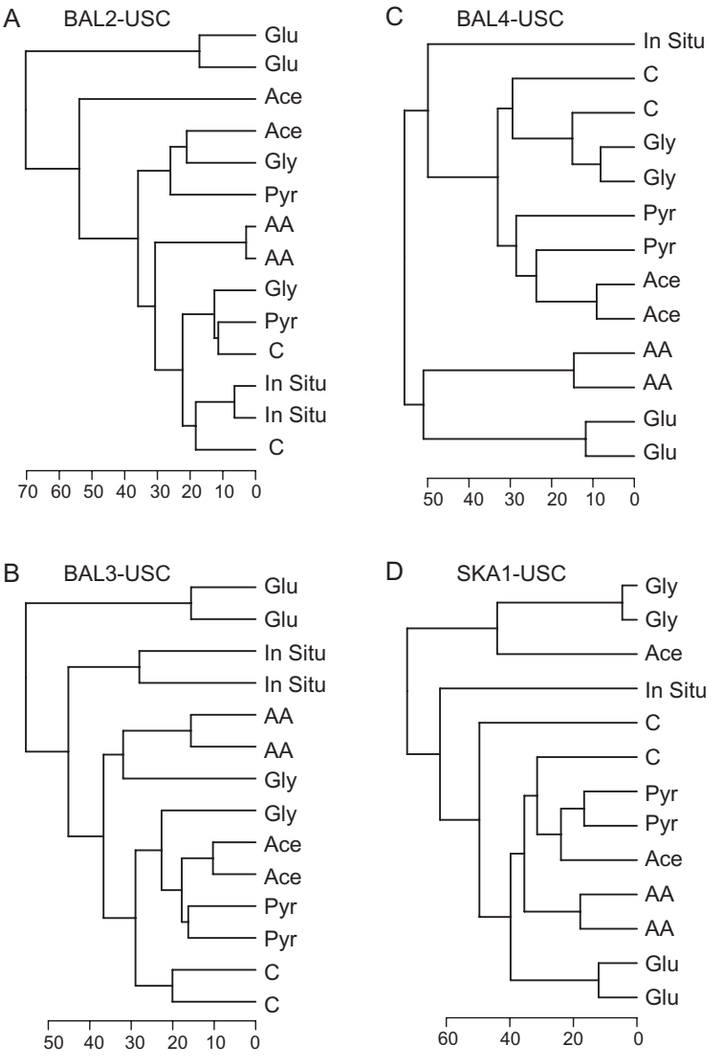

Fig. 4. Cluster analysis of bacterial community composition in the unfiltered seawater cultures (USC) enrichment experiments. (A) BAL2–USC, (B) BAL3–USC, (C) BAL4–USC and (D) SKA1–USC. The dendrograms are based on the analysis of the DGGE images in Fig. S2. Controls without enrichment (C), acetate (Ace), pyruvate (Pyr), glycolate (Gly), glucose (Glu) or amino acids (AA).

Taxon-specific responses to carbon compounds

The response of the bacterial community to the different carbon compound enrichments was evaluated by sequencing a total of 78 bands extracted from the different gels. The identity and phylogeny of the sequenced phylotypes are presented in Table 2 and Fig. 5.

Gammaproteobacteria was the major group containing most of the identified bacteria, and several different patterns of carbon compound preference were detected within this group (Table 2, Fig. 5A). Members of the genus *Alteromonas* showed diverse responses to the carbon compounds studied. Several *Alteromonas* phylotypes (e.g. M1-A6 and M2-A7) were found in all, or nearly all, treatments in different experiments. At the same time, other *Alteromonas* phylotypes were selectively stimulated by specific carbon compounds, like phylotypes M2-A5, M3-A4 or M2-A6 that were only detected with acetate, glucose or pyruvate respectively. *Vibrio* phylotypes were selectively stimulated with glucose (phylotype S1-U3 in the Skagerrak) or amino acids (phylotype M1-A5 in the Mediterranean Sea). The growth of *Psychromonas* phylotype B3-U1 (nearly identical to B4-U14) was strongly stimulated by glucose enrichments in all three USC experiments in the Baltic Sea. Although found as a strong band in experiment BAL4–USC, it was relatively weak in experiment BAL4–ASDC that was run in parallel. Phylotypes related to *Neptuniibacter caesariensis* (M2-A2, M2-A8 and M2-A4) responded positively, but differently, to the compounds acetate, pyruvate and DMSP (Fig. 5A). A relatively close relative, *Amphritea balenae* phylotype M3-A1, was only found with DMSP and amino acids. Bacteria belonging to the genus *Oceaniserpentilla* (phylotypes M1-A7 and M2-A3) were detected only in the acetate treatments in two independent experiments in the Mediterranean. In

Table 2. Identity of bacterial phylotypes found during the carbon compound enrichment experiments.

Phylotype	Accession number	Closest relative in GenBank (Accession Number; % identity)	*In situ*	Control	Treatment					
					Acetate	Pyruvate	Amino acids	Glucose	DMSP	Glycolate
		Gammaproteobacteria								
M1-A6	HQ836381	*Alteromonas addita* (AY682202; 96%)	–	+	+	+	+	+	+	ND
M2-A1	HQ836395	*Alteromonas addita* (AY682202; 97%)	ND	+	–	–	+	–	+	ND
M2-A7	HQ836401	Uncult. *Alteromonas* sp. clone C50-024 (AM941189; 99%)	ND	++	++	–	++	++	++	ND
M3-A7	HQ836409	Uncult. *Alteromonas* sp. clone C50-024 (AM941189; 90%)	ND	–	–	–	++	–	–	ND
M3-A2	HQ836405	Uncult. *Alteromonas* sp. clone C50-024 (AM941189; 99%)	ND	–	–	–	–	–	+	ND
M1-A4	HQ836379	Uncult. *Alteromonas* sp. clone C50-024 (AM941189; 97%)	–	+	+	+	+	+	–	ND
M1-A12	HQ836387	Bacterium clone MOLA_APR06_80 m1 (EU338367; 97%)	–	+	+	+	–	–	+	ND
M2-A10*		*Glaciecola pallidula* ACAM 615T (U85854; 95%)	ND	+	+	+	+	–	+	ND
M1-A1	HQ836376	*Alteromonas addita* (AY682202; 97%)	–	–	–	–	–	+	++	ND
M3-A17	HQ836417	Uncultured *Alteromonas* sp. clone T534deg47 (JN210714; 84%)	ND	++	+	+	–	++	–	ND
M2-A5	HQ836399	*Alteromonas genoviensis* strain I96 (FJ040187; 99%)	ND	–	+	–	–	–	–	ND
M3-A4	HQ836407	*Alteromonas macleodii* isolate R-28696 (AM885869; 100%)	ND	–	–	–	–	+	–	ND
M2-A6	HQ836400	*Glaciecola agarilytica* strain NO2 16S (DQ784575; 100%)	ND	–	–	+++	–	–	–	ND
M2-A11	HQ836403	*Glaciecola* sp. SD6 (FJ230843; 98%)	ND		–	–	–	+	–	ND
M3-A10	HQ836412	*Aestuariibacter aggregatus* strain WH169 (FJ847832; 100%)	ND	+	–	–	–	+	–	ND
M1-A3	HQ836378	Uncult. bacterium EPR4055-N3-Bc50 (EU491918; 97%)	–	–	–	–	–	+	+	ND
M1-A23	HQ836393	Uncultured bacterium EPR4055-N3-Bc88 (EU491947; 95%)	+	+	+	+	–	–	–	ND
M1-A10	HQ836385	*Methylophaga* sp. AM3Q (EU001739; 98%)	+	+	+	+	–	–	+	ND
M1-A5	HQ836380	*Vibrio* sp. enrichment culture GASA1 (FJ178085; 100%)	–	–	–	–	++	–	–	ND
S1-U3	HQ836446	*Vibrio lentus* (AJ278881; 97%)	–	–	+	+	+	+++	ND	–
B4-U14*		Sponge bacterium Zo10 (AY948377; 96%)	–	–	–	–	–	++	–	–
B3-U1	HQ836423	*Psychromonas* sp. BSw20972 (GU166137; 95%)	–	–	–	–	–	+++	ND	–
M1-A22	HQ836392	*Pseudoalteromonas* sp. KDW4 (EU930871; 99%)	–	–	–	–	–	++	–	ND
M2-A4	HQ836398	*Neptuniibacter caesariensis* MED92 (AY136116; 97%)	ND	–	+	+	–	–	–	ND
M2-A8	HQ836402	*Neptuniibacter caesariensis* MED92 (AY136116; 97%)	ND	–	–	–	–	–	++	ND
M3-A13	HQ836414	*Neptuniibacter caesariensis* MED92 (AY136116; 97%)	ND	–	–	–	–	–	+++	ND
M2-A2	HQ836396	Carbazole-degrading bacterium CAR-SF (AB086227; 97%)	ND	–	++	++	–	–	++	ND
M3-A16	HQ836416	*Marinobacterium* sp. J5 (AJ630166; 98%)	ND	+	+	+	–	–	–	ND
M3-A1	HQ836404	*Amphritea balenae* strain: JAMM 1525 (AB330883; 99%)	ND	–	–	–	++	–	++	ND
M3-A11	HQ836413	*Neptuniibacter caesariensis* MED92 (AY136116; 96%)	ND	+++	+++	–	–	–		ND
M1-A7	HQ836382	Uncult. Gammaproteobacterium PM1-27 (EF215799; 98%)	–	–	++	–	–	–		ND
M2-A3	HQ836397	Uncult. Gammaproteobacterium PM1-30 (EF215802; 98%)	ND	–	+	–	–	–		ND
M1-A14	HQ836388	Uncult. Gammaproteobacterium DPC012 (DQ269073; 94%)	–	–	–	–	–	+	–	ND
M3-A9	HQ836411	Uncult. Gammaproteobacterium DPC012 (DQ269073; 93%)	ND	–	–	–	–	+	–	ND
M1-A16	HQ836389	*Marinomonas blandensis* strain MED121 (DQ403809; 100%)	–	–	–	+	–	–	++	ND
M1-A2	HQ836377	*Marinomonas blandensis* strain MED121 (DQ403809; 100%)	–	–	–	++	–	–	++	ND
B4-A7	HQ836433	*Marinomonas* sp. BSw10506 (EF437161; 99%)	–	+	+	+	+	+	ND	+
B4-A10	HQ836436	*Marinomonas* sp. DG1602 (EU052766; 98%)	+	++	++	–	–	–	ND	++
M1-A19	HQ836390	Uncultured bacterium clone 006A8 (EU734915; 94%)	+	+	+	–	–	–	–	ND
M3-A22	HQ836418	*Alcanivorax venusti* strain ISO4 (AF328762; 100%)	ND	+	+	–	–	–	–	ND
B1-A3	HQ836422	*Acinetobacter lwoffii* strain S3-2 (FJ373029; 94%)	ND	–	+++	–	–	–	ND	ND
M1-A24	HQ836394	Uncult. bacterium clone ORCA-17N119 (DQ823221; 97%)	++	–	–	–	–	–	–	ND
		Alphaproteobacteria								
B4-A9	HQ836435	*Loktanella vestfoldensis* isolate IMCC6033 (EU687493; 98%)	–	–	–	–	+	–	ND	–
S1-U7	HQ836450	Uncult. Alphaproteobacterium M0-Ar2-P4F09 (EF016464; 99%)	+	–	–	–	–	–	ND	–
M3-A14	HQ836415	Uncult. marine bacterium clone AG-1 (DQ787776; 94%)	ND	+	–	–	–	–	–	ND

Table 2. *cont.*

Phylotype	Accession number	Closest relative in GenBank (Accession Number; % identity)	Treatment							
			In situ	Control	Acetate	Pyruvate	Amino acids	Glucose	DMSP	Glycolate
M3-A8	HQ836410	*Thalassobius mediterraneu*s CECT 5383 (AJ878874; 99%)	ND	–	–	–	+	–	–	ND
M3-A3	HQ836406	*Thalassobius mediterraneus* (AJ878874; 99%)	ND	–	–	+	+	–	–	ND
S1-U5	HQ836448	*Roseovarius crassostreae* CV919-312 (AF114484; 96%)	+	+	++	++	++	++	ND	+
S1-U1	HQ836444	Uncult. marine bacterium 16_03_05C02 (FR684249; 98%)	–	++	+	++	++	++	ND	+
S1-U2	HQ836445	Uncult. Alphaproteobact. GCHU11_A (AY701453; 98%)	–	–	–	–	–	–	ND	+++
B4-A6	HQ836432	*Rhodobacter ovatus* (AM690348.2; 90%)	–	+	+	+	+	+	ND	+
		Betaproteobacteria								
B1-A1	HQ836420	Uncult. Betaproteobacterium 4887-27F (FR648297; 96%)	ND	++	–	++	++	++	ND	ND
B1-A2	HQ836421	Uncult. Betaproteobacterium 4887-27F; FR648297; 99%)	ND	++	+	++	++	++	ND	ND
B3-U2	HQ836424	Betaproteobacterium BAL58 (AY317112; 99%)	–	+	+++	++	–	++	–	ND
B4-A1	HQ836428	*Limnobacter thiooxidans* (AJ289885; 99%)	+	++	+++	+++	+++	+++	ND	++
M1-A21	HQ836391	*Limnobacter thiooxidans* strain CS-K2 (NR_025421; 88%)	–	++	++	++			ND	
B4-U21*		Uncultured bacterium clone 3C003461 (EU802067; 81%)	–	–	–	–	++	–	ND	–
		Epsilonproteobacteria								
M1-A8	HQ836383	Uncultured bacterium B1_10.2_2 (FJ717102; 100%)	–	–	–	++	–	–	–	ND
		Bacteroidetes								
B4-A8	HQ836434	Uncult. Flavobacteria NorSea69 (AM279175; 97%)	–	–	+	+	+	+	ND	–
B3-U4	HQ836426	Uncult. Bacteroidetes CB41G0 (EF471632; 98%)	+	+	+	+	+	+	ND	+
M3-A23	HQ836419	*Lishizhenia caseinilytica* (AB176674; 93%)	ND	–	–	+++	–	–	–	ND
B4-A5	HQ836431	Uncultured Bacteroidetes DGGE band BP7 (DQ270277; 99%)	+	+	+	+	+	+	ND	+
B4-U10	HQ836437	Uncultured Bacteroidetes DGGE band BP7 (DQ270277; 99%)	+	+	+	+	+	+	ND	+
B4-U18	HQ836443	Uncultured Bacteroidetes DGGE band BP6 (DQ270276; 99%)	+	+	+	+	+	+	ND	+
B3-U3	HQ836425	Uncult. Bacteroidetes DGGE band BP2 (DQ270272; 99%)	+	+	+	+	+	+	ND	+
M1-A9	HQ836384	Uncultured bacterium clone S25_1310 (EF574966; 93%)	+	–	–	–	–	–	–	ND
B3-U3	HQ836425	Uncultured Bacteroidetes DGGE band BP2 (DQ270272; 99%)	+	+	+	+	+	+	ND	+
M3-A5	HQ836408	Uncult. Bacteroidetes XME60 (EF061974; 93%)	ND	–	–	–	–	+	ND	–
B4-U15	HQ836440	Uncult. Bacteroidetes DGGE band BP17 (DQ270287; 99%)	+	+	+	+	+	+	ND	+
B4-U11	HQ836438	Uncultured bacterium clone 5C231733 (EU804047; 99%)	+	+	+	+	+	+	ND	+
M1-A11	HQ836386	Uncultured bacterium clone Hg91A6 (EU236350; 93%)	–	+	+	+	–	–	+	ND
S1-U6	HQ836449	Uncultured Bacteroidetes bacterium CA-42 (EF419214; 98%)	+	+	++	+	+	+++	ND	+
B4-U16	HQ836441	Uncultured bacterium clone 3C002904 (HQ380670; 100%)	+	+	+	+	+	+	ND	+
		Actinobacteria								
B4-A4	HQ836430	Uncultured bacterium clone 5C231436 (EU803804; 96%)	+	–	–	–	–	–	ND	ND
B3-U5	HQ836427	Uncultured bacterium clone 5C231436 (EU803804; 92%)	ND	+	+	+	+	+	ND	+
B4-U17	HQ836442	Uncultured bacterium TG_FD0.2_W_AC01 (EU803804; 99%)	+	+	+	+	+	+	ND	+
B4-U13	HQ836439	Uncultured bacterium clone: S9D-06 (AB154303; 96%)	+	+	+	+	+	+	ND	+
		Cyanobacteria								
S1-U4	HQ836447	Uncultured *Synechococcus* sp. Mes1 (EF441554; 98%)	+	–	–	–	–	–	ND	–

Also indicated is the relative abundance of the phylotypes, as inferred from DGGE band intensities of the phylotypes in the different enrichments: (+) denotes abundant phylotypes and (–) denotes phylotypes not detected. ND, not determined, indicates that a specific carbon compound was not used in the experiment where a certain phylotype was originally identified. Identity of phylotypes was determined from analysis of the 16S rRNA gene sequences from excised DGGE bands. Phylotypes are ordered according to phylogeny, as deduced from the phylogenetic trees in Fig. 5. Phylotype codes designate the experiment from which the bands were excised (i.e. M1 for experiment MED1, M2 for MED2, M3 for MED3, B1 for BAL1, B3 for BAL3, B4 for BAL4 and S1 for SKA1). This coding allows localizing the different bands from which specific phylotypes were sequenced in the DGGE images from the different experiments in Figs S1 and S2. Asterisks (*) denote sequences of low quality, not submitted to GenBank.

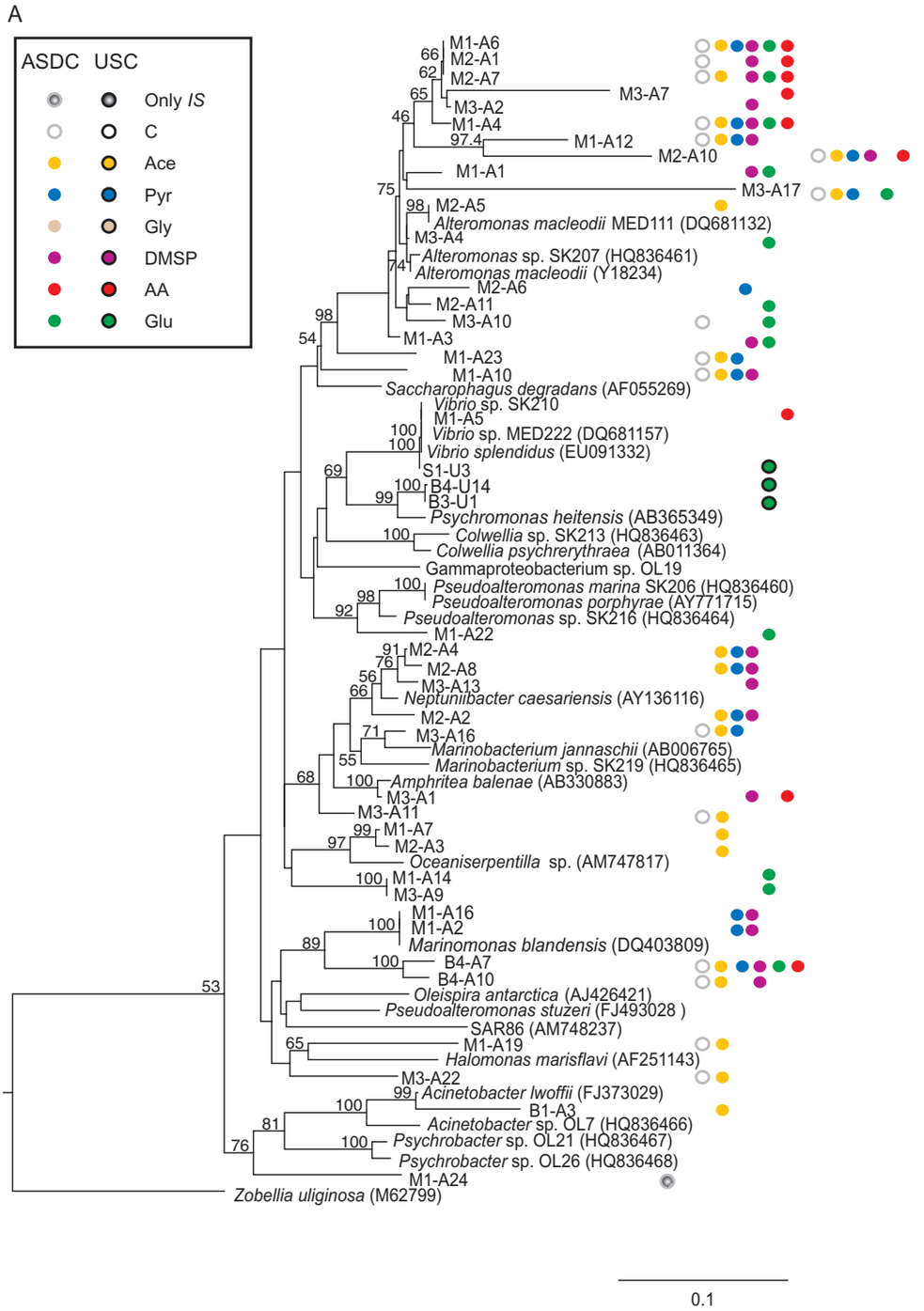

Fig. 5. Phylogenetic trees of 16S rRNA gene sequences retrieved from DGGE bands. (A) Gammaproteobacteria, (B) Alpha-, Beta- and Epsilonproteobacteria, (C) Bacteroidetes and Actinobacteria. Also shown are reference sequences from cultured bacteria. Colour-filled circles designate the presence of phylotypes in the enrichments. Circles with a black ring designate phylotypes retrieved from unfiltered seawater cultures. ClustalW was used for sequence alignment and neighbour joining for tree construction with the software MegAlign in the DNASTAR package (version 7.0). Numbers at nodes are bootstrap values after 1000 replicates. The scale bar represents the number of amino acid substitutions per position. Phylotype codes designate the experiment from which the bands were excised (i.e. M1 for experiment MED1, M2 for MED2, M3 for MED3, B1 for BAL1, B3 for BAL3, B4 for BAL4 and S1 for SKA1). This coding allows localizing the different bands from which specific phylotypes were sequenced in the DGGE images from the different experiments in Figs S1 and S2. Controls without enrichment (C), acetate (Ace), pyruvate (Pyr), glycolate (Gly), amino acids (AA), glucose (Glu), dimethylsulphoniopropionate (DMSP) and natural seawater (Nat. SW).

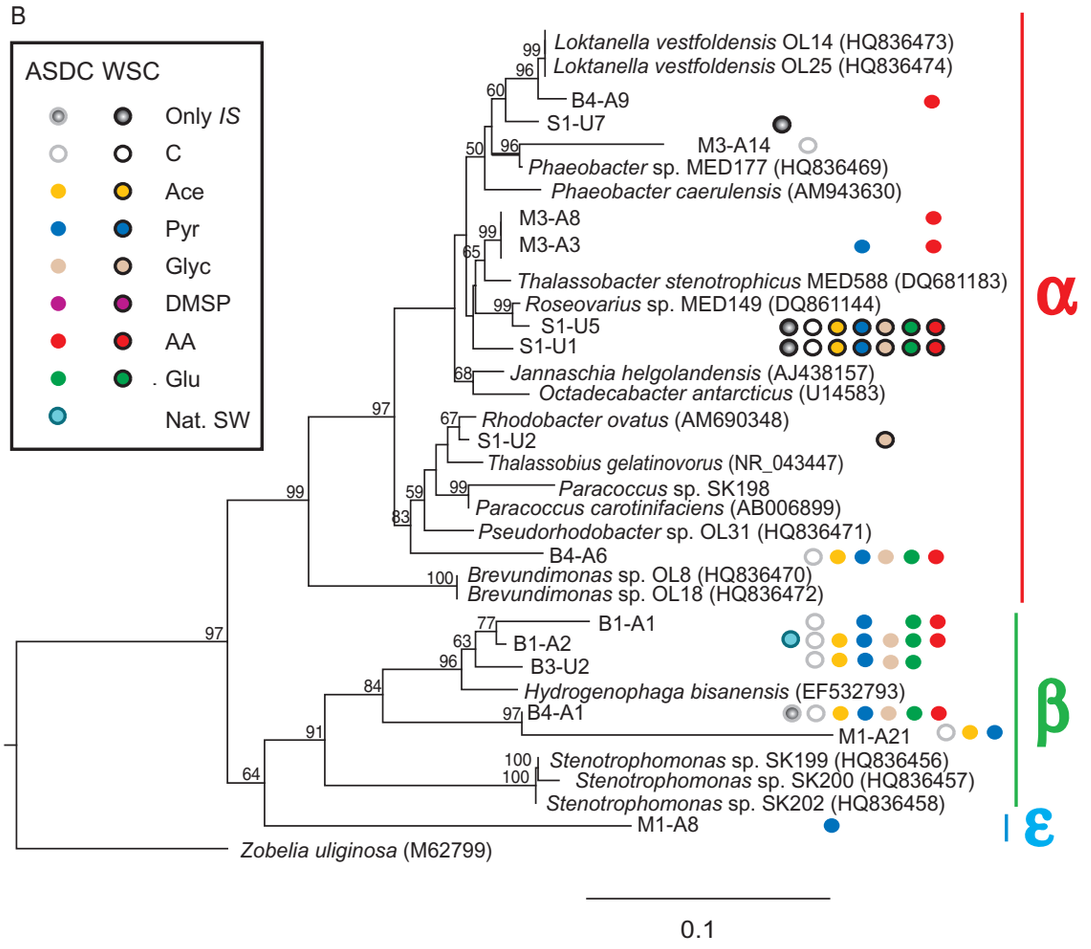

Fig. 5. *cont.*

two separate experiments, a novel Gammaproteobacteria taxon, represented by the phylotypes M1-A14 and M3-A9 in MED1–ASDC and MED3–ASDC, respectively, was selectively stimulated in only one replicate each of the glucose treatments. *Acinetobacter* phylotype B1-A3 was strongly stimulated in the acetate enrichments in one of the Baltic Sea experiments.

Eight identified phylotypes in our experiments belonged to the Alphaproteobacteria (Table 2, Fig. 5B). Two different phylotypes (*Loktanella* B4-A9 and *Thalassobius* M3-A8) responded in the amino acids enrichments only. However, *Thalassobius* phylotype M3-A3 (nearly identical to M3-A8) was identified in the pyruvate enrichment as well as in the amino acids. *Roseobacter* clade phylotypes S1-U1 and S1-U5 were found in all treatments, possibly indicating a relatively wide spectrum of carbon compound use. Phylotype S1-U2, a member of the Rhodobacteraceae family, was strongly stimulated with glycolate in the Skagerrak.

All Betaproteobacteria phylotypes identified in this study, except one (*Limnobacter* M1-A21 from the Mediterranean), were found in experiments in the Baltic Sea. The *Hydrogenophaga*-related phylotypes B1-A2, B3-U2 and B4-A1 became dominant in most of the treatments (Fig. 5B). However, M1-A21 was only present with acetate and pyruvate (Fig. S2A). Phylotype M1-A8 was the only member of the Epsilonproteobacteria found in our experiments and it became dominant in one of the pyruvate replicates of experiment MED1–ASDC (Table 2, Fig. S1).

A majority of the identified Bacteroidetes phylotypes in our experiments were present in most of the carbon compound enrichments (Table 2, Fig. 5C). However, three phylotypes showed specific responses: M3-A23 was found in pyruvate, M3-A5 in glucose and S1-U6 was dominant with acetate and glucose. Actinobacteria phylotypes were only found in the Baltic Sea experiments (i.e. B3-U5, B4-U17, B4-U13), where they appeared independently of the type of carbon compound enrichment.

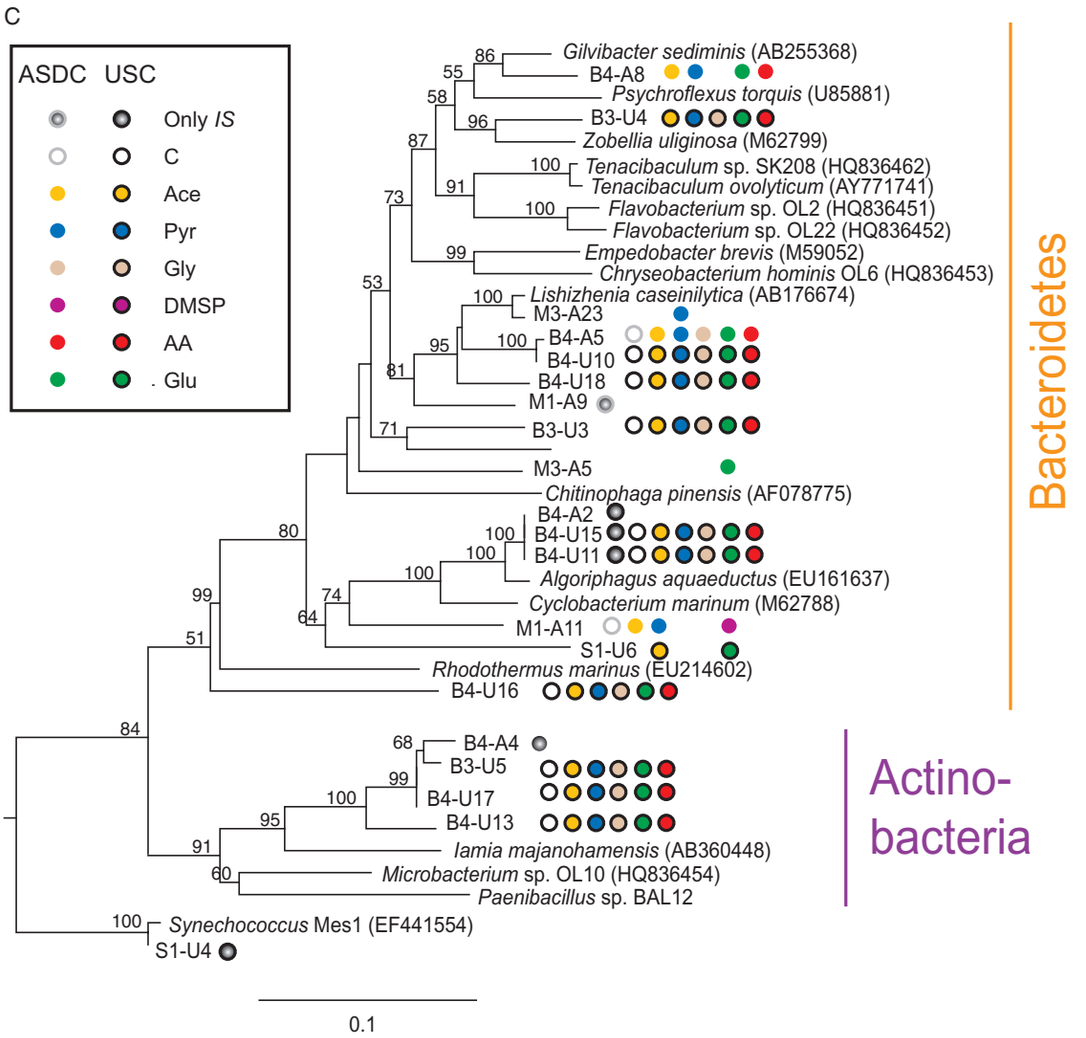

Fig. 5. *cont.*

Discussion

A variety of experimental approaches have shown that major groups of bacteria (e.g. Alphaproteobacteria versus Bacteroidetes) differ in their preference for different organic substrates (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Pernthaler *et al.*, 2002; Teira *et al.*, 2004; Vila *et al.*, 2004; Elifantz *et al.*, 2005; Alonso and Pernthaler, 2006; Alonso-Sáez and Gasol, 2007). Challenging these studies, a report by Mou and colleagues (2008) applying experimental metagenomics showed that, even though the addition of the distinct carbon compounds DMSP and vanillate stimulated the synthesis of new bacterial DNA, the phylotypes responding to these specific additions were basically the same. They thus suggested that bacterioplankton from coastal waters have a dominant generalist behaviour when consuming specific compounds of the DOC pool. Nevertheless, the short read length of the pyrosequencing method used, where only about one-tenth of each gene was covered by the metagenomic sequence data set, allowed only comparisons of the substrate-specific metagenomes at the taxonomical level of order. The authors therefore speculated that their interpretation could change if the bacterioplankton responses were analysed at a higher level of taxonomic resolution (Mou *et al.*, 2008). We used PCR-DGGE analysis to detect changes in bacterial community composition triggered by specific organic carbon compound enrichments. This allowed visualization of relative changes in bacterial community composition and identification of the dominant bacterial phylotypes through sequencing of bands (i.e. partial 16S rRNA gene sequences). The sequences obtained here had a length of about 500 bp, which permits the discrimination of dif-

ferent phylotypes (i.e. particular bacterial sequence types) within larger phylogenetic clusters (e.g. genera or families). Our results corroborate the finding of Mou and colleagues (2008) in that, at the level of class, and even at the level of order, marine bacterioplankton may be considered as generalists. For example, our finding of several individual phylotypes in the order Alteromonadales (class Gammaproteobacteria) responding to many carbon compounds would suggest they are generalists. At the same time, however, many phylotypes even within the genus *Alteromonas* were highly restricted to certain carbon compounds, suggesting that they are specialists. Together, our experiments showed that specific carbon compounds stimulated the growth of particular bacterioplankton members in many cases and that this was most evident at the species level.

According to our null hypothesis, based on the assumption that marine bacteria should be generalists with respect to the use of low-molecular-weight labile DOC compounds, we expected no differences in the banding pattern of DGGE fingerprints from bacterial assemblages that developed in seawater cultures enriched with different monomeric carbon compounds. Indeed, in a few experiments, we did find individual dominant phylotypes that occurred in all carbon compound enrichments, indicating that they had a broad spectrum of utilization of different labile organic carbon compounds. This was particularly pronounced among the Bacteroidetes and Actinobacteria phylotypes in the Baltic Sea experiments. However, the null hypothesis as a whole should be rejected on two accounts. First, in nearly all experiments there was at least one of the carbon compounds investigated that resulted in the increase in relative abundance of one individual phylotype that was characteristic for that specific carbon compound. Examples of this pattern of distribution were the dominance of *Acinetobacter* phylotype B1-A3 in the acetate enrichment in experiment BAL1–ASDC, *Psychromonas* phylotype B3-U1 in the glucose enrichment in experiment BAL3–USC and *Rhodobacter* phylotype S1-U2 in experiment SKA1–USC. Second, in several experiments different carbon compounds triggered the development of unique combinations of dominant phylotypes, although individual phylotypes were not necessarily unique to each carbon compound. This was most prominent in experiment SKA1–USC. Rejection of our null hypothesis implies that not all marine bacterioplankton are generalists. Rather, our data show that several individual bacterial taxa respond differently to labile organic compound inputs, suggesting that under certain ecological conditions they could effectively be considered organic matter specialists.

In the present study we used a subset of different labile monomeric/low-molecular-weight carbon compounds known to be substrates of importance to marine bacteria (Kujawinski, 2011). In the sea, the concentration of these compounds is highly variable on temporal and spatial scales, as is the measured bulk bacterial uptake of the same compounds (Fuhrman, 1987; Suttle *et al*., 1991; Rich *et al*., 1996; Obernosterer *et al*., 1999; Skoog *et al*., 1999; Ho *et al*., 2002; Simó *et al*., 2002). Thus, if bacteria differ in their capacity and/or efficiency in utilizing such compounds for growth and respiration (e.g. by having transport systems with different affinities for a certain compound), the availability of resources could be important in structuring bacterial community composition. However, such a conclusion would critically depend on how we perceive the metabolic capacity of bacteria in relation to the availability of specific organic compounds in the sea. Our results indicate that marine bacteria can be classified into different tentative categories with respect to how they benefit from substrate inputs when growing in the presence of other bacteria (i.e. not in monoculture): (i) strict specialists, i.e. bacteria responding to only one carbon compound, (ii) moderate specialists, bacteria responding to a few carbon compounds that are likely to be abundant in certain situations (e.g. compounds released by phytoplankton) and (iii) generalists, bacteria that respond to many and structurally different carbon compounds. These categories should be interpreted with caution, since it is unlikely that any strict specialist bacterium would be unable to use no more than one single compound if given the chance, or that a true generalist could consume all possible available compounds. We find it highly likely that whether or not an individual bacterial species behaves as a strict or moderate specialist would depend on the presence of other taxa in its vicinity (i.e. through direct competition for resources). Moreover, even if a species has the metabolic machinery to utilize certain compounds, factors like substrate concentration (e.g. Alonso and Pernthaler, 2006) or the particular physical, chemical or biological context (e.g. temperature, inorganic nutrient availability or accompanying microbial assemblage) will influence the ability of bacteria to use them.

We identified a substantial diversity of phylotypes with preferences for different carbon compounds. As previously observed, Gammaproteobacteria such as *Vibrio* and *Alteromonas* spp. thrived in seawater experiments with allochthonous carbon enrichments, and consequently they are frequently referred to as copiotrophs, 'weeds' or opportunists (Fuchs *et al*., 2000; Allers *et al*., 2007). Indeed, vibrios have a series of genome properties typical for extreme copiotrophs, such as many rRNA operons (Lauro *et al*., 2009). However, and somewhat unexpected, we note that: (i) not all phylotypes that responded to the investigated carbon compounds were known copiotrophic bacteria and (ii) even among bacterial groups known as copiotrophs, there were, in addition to generalist phylotypes, particular specialist phylotypes that

responded only to specific carbon compounds. For example, while some *Alteromonas* phylotypes reacted universally to manipulation (i.e. phylotypes were also detected in controls; like M2-A1, M2-A7, M1-A4), other members of that genus needed supplementary carbon compounds in order to grow (e.g. phylotypes M2-A5, M2-A6, stimulated by different carbon compounds). Tentatively defining as generalist bacteria those phylotypes that used four or more out of five carbon compounds in each experiment (not counting the controls), and specialists those that used only one compound, resulted in the emergence of some interesting preference patterns. Thus, across all our experiments, among the 42 phylotypes in the Gammaproteobacteria that showed a response, only five phylotypes would classify as generalists. On the other hand, 21 out of 42 phylotypes appeared to be specialists. This suggests that among the Gammaproteobacteria there is a large diversity of specialists.

Concerning other Gammaproteobacteria phylotypes that became dominant in specific enrichments, some deserve brief mentioning regarding what is known about them. A few phylotypes preferentially thrived with acetate, such as *Acinetobacter* phylotype B1-A3 in experiment BAL1–ASDC. Members of the genus *Acinetobacter* are ubiquitous in both soil and aquatic environments, and are known to be competitive in experiments with acetate as sole carbon compound for growth (Towner *et al*., 1991). Incidentally, microbial uptake of radiolabelled substrates measured *in situ* showed remarkably high assimilation rates of acetate on the day the water for this experiment was collected (data not shown). *Marinomonas* phylotype M1-A2, found to be dominant with pyruvate and DMSP, had a partial 16S rRNA gene sequence identical to the whole-genome sequenced bacterium *Marinomonas blandensis* strain MED121. Screening of this genome revealed the *dmdA* and phosphoenolpyruvate synthetase genes, which are necessary for utilization of DMSP and pyruvate as carbon sources for growth respectively (Postma and Roseman, 1976; Howard *et al*., 2006).

Overall, although a wide diversity of bacteria thrived in the experiments, it should be noted that the organisms that responded to carbon enrichments are not necessarily the organisms that are oxidizing the bulk of the same substrates in nature. For example, bacteria in the SAR11 clade are important in the processing of DMSP in oceanic waters (Malmstrom *et al*., 2004), but are rapidly outcompeted in enrichment experiments like ours, potentially due to too high substrate concentrations for these oligotrophic bacteria. Moreover, an experimental approach like the one used here, using enrichments with single (albeit ecologically relevant) model carbon compounds, should not be expected to elicit the same response by the bacterioplankton as the response to variation of particular compounds against a background of various labile or complex DOC compounds in natural waters. Future work linking the abundance of specific bacterial taxa to variations in particular carbon compounds (determined for example through mass spectrometry and/or nuclear magnetic resonance approaches) will have the potential to show if bacterial responses as those found in our study are also distinguishable in the sea. It is also likely that some bacteria need essential cofactors (e.g. vitamins or iron) to compete successfully for particular carbon compounds, and that the lack of such factors limited the response of bacteria in the experiments. We used enrichments corresponding to the micromolar concentrations of total labile DOC found in the coastal waters studied, while in the sea the particular compounds used vary at nanomolar concentrations. The high concentration could favour organisms that grow rapidly and would not reveal the competition for substrates that occurs at nanomolar substrate concentrations. These considerations are important when interpreting our results, together with the notion that our study was not designed to study the specialized carbon cycling activities of bacterioplankton in their natural environment.

We did enrichment experiments using both unfiltered seawater with unmanipulated microbial assemblages and seawater dilution cultures where flagellate grazers had been removed by filtrations. Indeed, the bacterial abundance results and leucine incorporation data in Figs 1 and 2 showed that grazing was important in the unfiltered treatments and had consequences for the development of bacterial populations in the experiments. The distribution of *Psychromonas* phylotype B3-U1 could serve as an example: in experiment BAL4–ASDC without grazing, this phylotype (represented by band B4-A8 in Fig. S1E) was present at relatively low abundance with acetate, amino acids and glucose. However, in both experiments BAL3–USC and BAL4–USC (with grazing maintained) this phylotype (represented by band B3-U1 or B4-U14 in Fig. S2B or C respectively) became dominant in the glucose treatment only. This suggests that this *Psychromonas* species is a competitive glucose scavenger primarily when grazers are present. This example indicates that the success of specific bacterial taxa may differ between experimental approaches (due to differences in growth conditions with or without grazers). Still, and independently of experimental approach, our collective results substantiate that the availability of particular carbon compounds is important for structuring bacterioplankton communities by selecting for specific phylotypes or distinct sets of bacteria.

This experimental study with coastal seawater, and the natural bacterioplankton therein, was carried out to examine the potential of particular carbon compounds to select for specific organisms or sets of organisms. We found that, in most experiments, both generalist and

specialist bacterial populations developed in response to different carbon compound enrichments, suggesting that both strategies can be important for defining bacterial ecosystem functioning characteristics. The results indicate the feasibility of investigating microbial assemblages as well as specific model organisms to understand how the interactions between particular organic compounds and bacteria determine the ecology and biogeochemistry of microbial carbon cycling in the ocean. In particular, it would be desirable to understand the molecular mechanisms regulating carbon cycling through specific DOC compounds of different classes (e.g. organic acids, sugars and amino acids). The experimental data presented here, in combination with *in silico* data increasingly available from genome sequencing of marine bacteria (Konstantinidis *et al*., 2009; Lauro *et al*., 2009; Newton *et al*., 2010), strongly suggest that different bacterial taxa, even those of the same order or genus, have different ecological roles in the transformation of the marine dissolved organic matter pool.

Experimental procedures

Seawater cultures

Two different experimental setups based on seawater cultures (i.e. ASDC and USC, see below), were carried out in a total of nine experiments with seawater from the Mediterranean Sea (Blanes Bay Microbial Observatory), the Baltic Sea and the Skagerrak in the eastern North Sea (Table 1).

Aged seawater dilution cultures were designed to study the effect of enrichment of specific carbon compounds on the bacterioplankton community when no other labile DOC compounds or predators were present. To prepare seawater medium of low labile DOC concentrations, surface water from each sea was collected and stored for approximately 1 year at room temperature in the dark. During this period of time, bacteria in the stored seawater were expected to consume the labile DOC. At the time of the experiments, aged seawater to be used as growth medium was filtered through 0.2 μm pore size Sterivex filter capsules (Millipore) using a peristaltic pump. This filtered water was distributed in 2 l polycarbonate bottles (Nalgene; 1900 ml each) and inoculated with a final concentration of 5×10^4 bacteria per millilitre (representing approximately a 20-fold dilution of the inoculum). The inoculum was prepared by gravity filtration of freshly sampled water through a 47 mm diameter 0.8 μm pore size (0.6 μm for BAL4–ASDC) polycarbonate filters (Nuclepore).

Unfiltered seawater cultures were used to analyse the consequences of specific carbon compound additions on the bacterioplankton community when nearly the entire microbial food web (including grazers) and the natural organic matter were present. For the USC, 1.5 l of surface seawater was transferred to acid- and sample water-rinsed polycarbonate bottles (Nalgene).

All seawater cultures (USC and ASDC) were maintained at *in situ* temperatures with the exception of BAL4–ASDC, BAL4–USC and SKA1–USC, which were incubated 5°C above the *in situ* temperature. This was done to reduce potentially confounding impacts of lower temperature on bacterial activities and carbon compound utilization patterns. Cultures were incubated in the dark (to avoid the potential effects on bacteria of organic matter production from phytoplankton photosynthesis or bacterial phototrophy) and monitored during approximately 5 days. Duplicate carbon-enriched treatments received a final concentration of 20 μM carbon of either of the specific carbon compounds. Assuming an average labile DOC concentration of 20 μM in the studied waters (Zweifel *et al*., 1993; J. Pinhassi, unpublished), these enrichments corresponded to a replenishment of the labile DOC pool by the specific carbon compounds. The compounds used were D(+)-glucose, L-amino acids, glycolate ($C_2H_4O_3$), acetate ($C_2H_3O_2Na \times 3H_2O$), pyruvate ($C_3H_3O_3Na$) and DMSP (see Table 1). The amino acid enrichment contained an equal mix of: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine. Carbon compounds were purchased from Sigma-Aldrich, except DMSP that was kindly provided by Rafel Simó (ICM-CSIC, Barcelona, Spain). An additional treatment using 0.2 μm filtered freshly sampled seawater medium was included in experiment BAL1–ASDC to determine bacterial growth on the natural DOC pool. Duplicate USC or ASDC controls received no carbon compound additions. All utensils in contact with the samples were acid-rinsed with 1 M HCl and extensively rinsed with Milli-Q water and sample water prior to use.

Bacterial abundance

Bacteria were enumerated by flow cytometry. Samples were preserved with a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and stored frozen at −70°C. Cell counts were carried out with a BectonDickinson FACSCalibur flow cytometer after the cells were stained either with Syto13 at a final concentration of 2.5 μM or SYBRGreen I at a 1:10 000 dilution of the commercial solution (Gasol and del Giorgio, 2000). Bacterial samples in experiment BAL1–ASDC were fixed with 0.2 μm pore size filtered formaldehyde (4% final concentration), stained with SYBRGold (1:100 dilution, Molecular Probes), filtered onto black 0.2 μm pore size polycarbonate filters (Poretics, Osmonics) and counted by epifluorescence microscopy within 48 h.

Bacterial activity

Bacterial activity was measured using the [^{3}H]-leucine incorporation method (Kirchman *et al*., 1985), as subsequently modified (Smith and Azam, 1992). Samples were collected after 2 days of incubation in experiments BAL2–USC and BAL3–USC, and after 3 days in BAL4–USC and SKA1–USC (i.e. at the days of sampling for DNA). For each sample, triplicate aliquots (1.2 ml) and a trichloroacetic acid-killed control were incubated with 40 nM [^{3}H]-leucine (final concentration; experimentally determined to be saturating in the studied waters) for 1 to 1.5 h at the experiment temperature in darkness.

Microbial community DNA

For collection of microbial community DNA, a 500–750 ml sample of each culture was filtered through a 0.2 μm pore size polycarbonate filter at < 200 mmHg to collect bacterial cells. For experiments MED1, MED2, MED3 and BAL1, community DNA was collected after 5 days' incubation. For the other experiments, community DNA was collected when the highest abundances were reached for each experiment, (approximately 48 h for BAL2–USC, BAL3–USC and SKA1–USC, 72 h for BAL4–USC and B4). Filters were immediately frozen at −70°C in TE buffer until further processing.

Microbial community DNA was obtained by phenol–chloroform extraction, following the protocols of Schauer and colleagues (2003) or Boström and colleagues (2004) for samples from the Mediterranean Sea or the Baltic Sea and Skagerrak respectively. DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantified fluorometrically (PicoGreen; Molecular Probes). Bacterial 16S rDNA was amplified by PCR using a bacterial primer complementary to position 341–358 with a 40 bp GC-clamp (GC341F; Muyzer *et al*., 1993) and a universal primer complementary to position 907–927 (907RM; Muyzer *et al*., 1998). Initial denaturation was at 95°C for 2 min followed by a thermal cycling programme as follows: denaturation for 30 s at 94°C; annealing for 30 s at an initial 63°C, decreasing 1°C every two cycles to a final of 53°C; extension for 90 s at 72°C. Ten cycles were run at 53°C for a total of 30 cycles followed by final 7 min of incubation at 72°C. The quality and size of PCR products were verified by agarose gel electrophoresis.

Denaturing gradient gel electrophoresis and sequencing

Sixty nanograms of PCR product were analysed by DGGE on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 29% to 52% in the D Gene System (Bio-Rad) at 60°C for 6 h at 150 V in 1× TAE (40 mM Tris, Acetic Acid, EDTA, pH 7.4). DGGE fingerprints were used to construct dendrograms using the package Vegan in R 2.12.0 (Oksanen *et al*., 2010) applying the Lance–Williams coefficients, Euclidean distances and unweighted-pair group means analysis (UPGMA).

Denaturing gradient gel electrophoresis bands were excised using a sterile razor blade and eluted in 20 ml of MilliQ water overnight at 4°C, followed by a freeze–thaw cycle. A total of 5 μl of the eluate was used for re-amplification with the original primer set. A part of the PCR product was analysed by DGGE together with the original sample to verify the correct position of the band, and in cases where more than one band was present the target band was processed again as described above. PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen) and quantified fluorometrically (PicoGreen; Molecular Probes) and subsequently sequenced by Macrogen (Korea). Sequenced DGGE bands (i.e. phylotypes) were defined as responsive to specific carbon compounds based on presence/absence or when band intensities in one or several treatments were more than five times stronger compared with controls or other treatments. Sequences have been deposited in GenBank under the Accession Numbers HQ836376–HQ836450.

Acknowledgements

We thankfully acknowledge the skilful technical assistance of Sabina Arnautovic and Vanessa Balagué. The insightful comments and constructive criticism on the manuscript by three anonymous reviewers is much appreciated. This work was supported by the Swedish Research Council, the Crafoord Foundation and the Linnaeus University Centre for Ecology and Evolution in Microbial Model Systems (EEMiS) to J. P., and by postdoc Grant No. 623-2009-7258 from the Swedish Research Council to L. G.-C. Sampling and experiments at Blanes Bay Microbial Observatory was supported by Spanish projects SUMMER (CTM2008-03309/MAR) and STORM (CTM2009-09352/MAR) to J. M. G.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DGGE fingerprints of bacterial assemblages in the aged seawater dilution culture (ASDC) enrichment experiments. (A) MED1, (B) MED2, (C) MED3, (D) BAL1, (E) BAL4. Controls without enrichment (C), acetate (Ace), pyruvate (Pyr), glycolate (Gly), amino acids (AA), glucose (Glu), dimethylsulphoniopropionate (DMSP) and natural seawater (Nat. SW). Several phylotypes in experiment MED1–ASDC were unique for one or a few enrichments (E; see also A–C and Table 2). For example, *Oceaniserpentilla* phylotype M1-A7 was unique for the acetate treatment while *Alteromonas* phylotype M1-A4 was only found with amino acids. *Alteromonas* M1-A1 was important only in DMSP (note that this band was different from *Limnobacter* phylotype M1-A21). Some phylotypes occurred in several, but not all, treatments, e.g. *Limnobacter* M1-A21 that was dominant in the control, acetate and pyruvate treatments or the phylotypes *Methylophaga* M1-A10, *Glaciecola* M1-A11 and *Alteromonas* M1-A12 that were present in all treatments except amino acids and glucose. Comparison of the banding pattern between the enrichment cultures and the *in situ* bacterial community revealed little, if any, overlap in dominant phylotypes (A). Also

in experiment MED2–ASDC there were phylotypes restricted to single treatments, such as *Glaciecola* M2-A6 that was strongly stimulated in the pyruvate treatment. Others, like *Neptuniibacter* phylotypes M2-A2 and M2-A4, were dominant in one or more replicates of DMSP, pyruvate and acetate treatments (B, Table 2), while *Alteromonas* phylotype M2-A7 was present both in the controls and in all treatments (except pyruvate). In experiment MED3–ASDC, *Aestuariibacter* phylotype M3-A10 was only detected in controls and with glucose and Bacteroidetes phylotype M3-A23 was only found in one of the pyruvate duplicates (C), while *Amphritea* phylotype M3-A1 (Oceanospirillales) was present in both DMSP and amino acid enrichments. In experiment BAL4–ASDC (E), the majority of phylotypes were present in most of the treatments; the main differences were related to band intensity rather than to band presence/absence (e.g. *Limnobacter* B4-A1 and Bacteroidetes B4-A5). *Marinomonas* phylotype B4-A10 was found in the control, glycolate and acetate, while *Loktanella* B4-A9 was restricted to amino acids.

Fig. S2. DGGE fingerprints of bacterial assemblages in the unfiltered seawater culture (USC) enrichment experiments. (A) BAL2–USC, (B) BAL3–USC, (C) BAL4–USC, (D) SKA1–USC. Abbreviations same as in Fig. S1. In experiments BAL2–USC and BAL3–USC (A and B respectively), *Psychromonas* phylotype B3-U1 strongly dominated in the glucose enrichments. Betaproteobacteria phylotype B3-U2 was found with different band intensities in all enrichments, except the amino acids. In experiment BAL4–USC (C), many of the phylotypes that were present in the *in situ* sample were also found in the enriched cultures. Compound-specific phylotypes were *Psychromonas* B4-U14 that was dominant with glucose, and *Marinomonas* B4-U20 and *Hydrogenophaga* B4-U21 that were found in the amino acids enrichment (see also Table 2).

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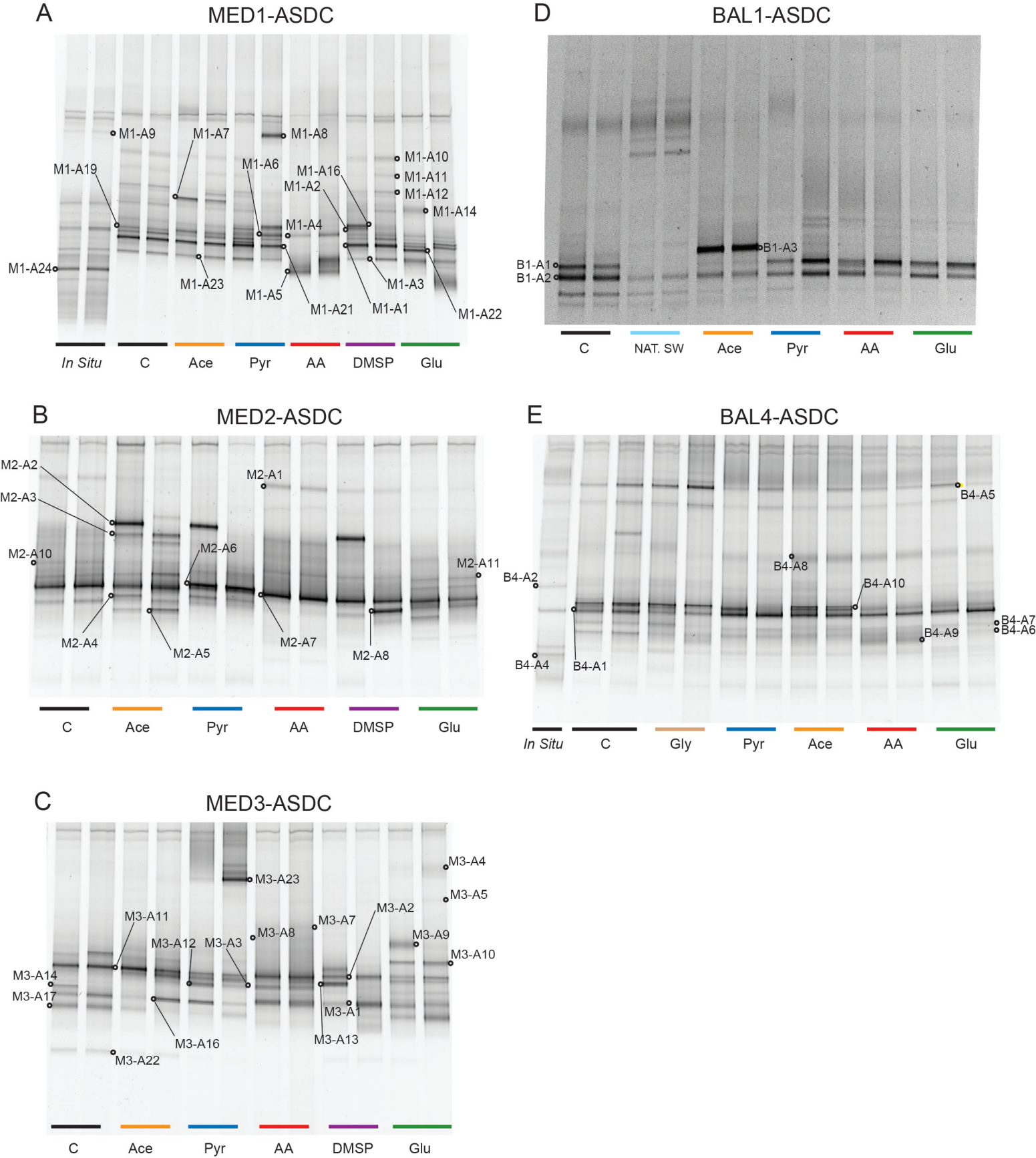
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M1-A21
M1-A1
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C
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BAL1-ASDC
B1-A3
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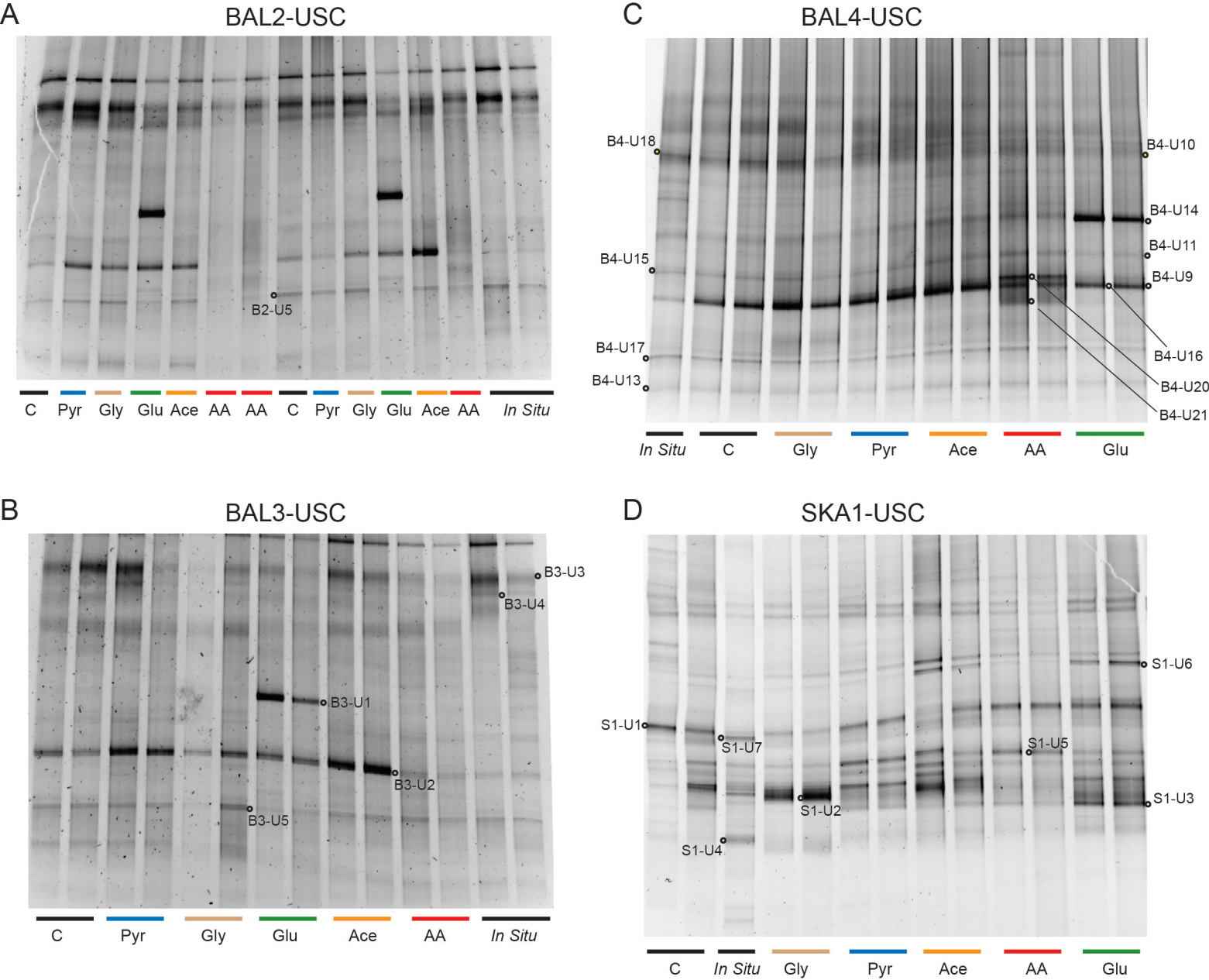
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BAL2-USC
B2-U5
C Pyr Gly Glu Ace AA AA C Pyr Gly Glu Ace AA In Situ
B
BAL3-USC
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B3-U2
B3-U5
C Pyr Gly Glu Ace AA In Situ
C
BAL4-USC
B4-U18
B4-U15
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B4-U13
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